



# Comparisons of Endothelial Cell G- and F-Actin Distribution in Situ and in Vitro

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**ABSTRACT.** Numerous studies have described the F-actin cytoskeleton; however, little information relevant to G-actin is available. The actin pools of bovine aortic endothelial cells were examined using in situ and in vitro conditions and fluorescent probes for G-(deoxyribonuclease I, 0.3  $\mu$ M) or F-actin (phalloidin, 0.2  $\mu$ M). Cells in situ displayed a diffuse G-actin distribution, while F-actin was concentrated in the cell periphery and in fine stress fibers that traversed some cells. Cells of subconfluent or just confluent cultures demonstrated intense fluorescence, with many F-actin stress fibers. Postconfluent cultures resembled the condition in situ; peripheral F-actin was prominent, traversing actin stress fibers were greatly reduced and fluorescent intensity was diminished. Postconfluency had little influence on G-actin, with only an enhancement in the intensity of G-actin punctate fluorescence. When postconfluent cultures were incubated with cytochalasin D (15 min;  $10^{-4}$  M), F-actin networks were disrupted and actin punctate and diffuse fluorescence increased. G-actin fluorescence was not altered by the incubation. Although its unstructured nature may account for the minor changes observed, the stability of the G-actin pool in the presence of notable F-actin modulations suggested that filamentous actin was the key constituent involved in these actin cytoskeletal alterations. A separate finding illustrated that the concomitant use of actin probes with image enhancement and fluorescent microscopy could reveal simultaneously the G- and F-actin pools within the same cell.

**Key words:** endothelial cells, actin, phalloidin, DNase

There are several pools of actin within nonmuscle cells (Korn 1982). Polymerized actin comprises the filamentous pool (F-actin), while monomeric actin forms the globular pool (G-actin). In addition, F-actin interacts with other contractile proteins to form complexes known as stress fibers. A number of qualitative studies have defined the distribution of F-actin and stress fibers in vascular endothelial cells under various conditions (Gabbiani et al. 1983, Gabbiani et al. 1975, Kalnins et al. 1981, Welles et al. 1985a,b, White et al. 1983, White and Fujiwara 1986, Wong et al. 1983). However, there is little qualitative information concerning F-actin and stress fiber arrangement relative to the distribution of G-actin. This information is necessary to verify quantitative findings and to understand better the degree of interaction among the various pools of actin. Such an understanding is important, since actin cytoskeletal changes are reported to influence such diverse endothelial cell activities as motility (Bottaro et al. 1985), permeability (Bottaro et al. 1986, Welles et al. 1985a,b) and metabolism (DuBose et al. 1987, 1989).

A widely used approach for the visualization of the F-actin pool employs the capacity of a fluorescent labeled mushroom toxin, phalloidin, to bind specifically with polymerized actin (Weiland and Faulstich 1978). In this report, a similar approach has been employed to observe G-actin. Deoxyribonuclease I (DNase I) interacts with this protein (Hitchcock 1980, Mannherz et al. 1980) and when conjugated with a flu-

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orescent element, DNase I can serve as a probe for G-actin. The current study describes the distribution of the actin pools in bovine aortic endothelial cells (BAEC) in situ and in vitro.

## MATERIALS AND METHODS

The in situ preparations were obtained as described previously (Rogers and Kalnins 1983a). Bovine aortas were cleaned of adventitia, rinsed in Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY), treated with 100% methanol ( $-20^{\circ}\text{C}$ ; 4 min) and then with 100% acetone ( $-20^{\circ}\text{C}$ ; 2 min). After fixation, the vessels were washed in HBSS, cut into  $0.5\text{ cm}^2$  sections and dried in a stream of freon. Next, a section was placed, endothelium side down, on a coverslip that was positioned on an aluminum plate cooled ( $-60^{\circ}\text{C}$ ) in a dry ice/ethanol bath. When ice crystals formed in a water drop placed near the segment, the segment was pried free to leave the endothelium attached to the coverslip.

The in vitro culture of BAEC employed modifications of procedures described previously (Jaffe et al. 1973). The aorta was rinsed in HBSS and a lateral cut was made to expose the tunica intima. A sterile scalpel blade was used lightly to scrape the endothelial cells from the vessel. Scrapings were deposited in collagenase (1 mg/ml; Cooper Biomedical, Malvern, PA) and incubated at  $37^{\circ}\text{C}$  for 10 min. Four milliliters of supplemented (10% fetal calf serum, penicillin:streptomycin (100 units/ml:100  $\mu\text{g}/\text{ml}$ ) and glutamine (0.29 mg/ml); GIBCO) Dulbecco's modified Eagle's Medium (DMEM; GIBCO) were then added. The cell suspension was centrifuged ( $3000 \times g$  for 5 min). The fluid was aspirated, the pellet suspended in supplemented DMEM (5 ml) and the solution pipetted into a tissue culture flask (Falcon 3013; Becton Dickinson, Lincoln Park, NJ). Cells were maintained on supplemented DMEM in a 5%  $\text{CO}_2$  incubator until they reached confluency (day 6). The culture was then rinsed with HBSS and the cells removed with 0.3 ml trypsin solution (0.5 mg/ml;

GIBCO). Cell number was determined and serial dilutions in supplemented DMEM were made to obtain a final dilution of 10 cells/ml. A 0.5 ml volume was placed in each well of a 24-well tissue culture plate (Falcon 3047; Becton Dickinson). In addition to supplemented DMEM, these cells were maintained with endothelial cell growth supplement (30  $\mu\text{g}/\text{ml}$ ; Collaborative Research, Bedford, MA) until small patches of growth were discernable by microscopic examination. After this point, cells were maintained with supplemented DMEM only. Cultures were observed for 2 weeks after obtaining confluency to ensure that they were free of smooth muscle contaminants, after which they were removed with trypsin, seeded into tissue culture flasks and grown to confluency. Cells were then harvested, resuspended at a final concentration of  $1 \times 10^6$  cells/ml supplemented DMEM and stored in an environment of liquid nitrogen until needed. From this stock, seedings were made on glass coverslips at a density of  $1.5 \times 10^2$  cells/ $\text{cm}^2$ . After culture for 3, 6, 10, 20 or 32 days, the cells were fixed in 3.7% formalin as described previously (Welles et al. 1985a,b). In other studies, post-confluent cultures (day 9) were treated with cytochalasin D ( $10^{-4}\text{ M}$ , 15 min) to alter F-actin arrangement and then fixed with formalin.

Rhodamine conjugated to phalloidin (R-P) or to DNase I (R-DNase I) (Molecular Probes, Eugene, OR) served as probes to delineate the F- and G-actin pools, respectively. To permit probe entry, the endothelial cell membranes were permeabilized with 80% acetone (10 min) as described previously (Welles et al. 1985a,b). Cells were then treated with R-P (0.2  $\mu\text{M}$ ) or R-DNase I (0.3  $\mu\text{M}$ ) for 10 min, after which the cultures were washed with HBSS five times. A Zeiss microscope equipped with a  $40\times$  water immersion objective and filters appropriate for rhodamine excitation was used to observe the cell fluorescence. Photographs of the fluorescence were taken using Tri-X Pan film (Kodak; 400 ASA). Automatic exposure times were employed for the photographs of BAEC in situ. When

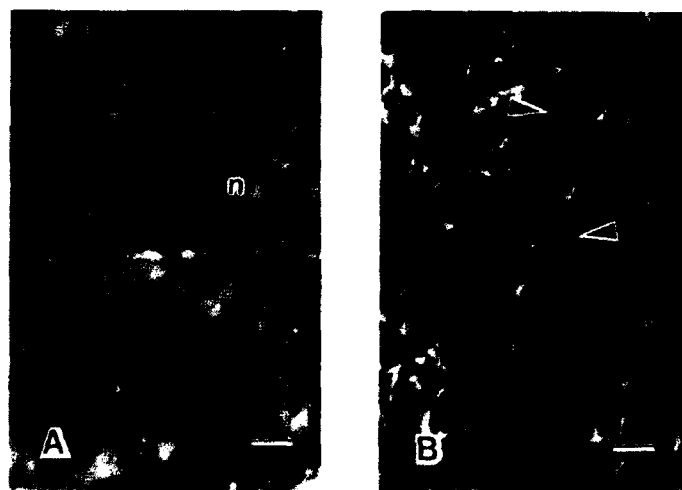
in vitro postconfluency state was studied, the same film exposure time was used that was found to be optimal for cultures at their first day of confluency (day 6). Exposure time for print development was also the same as that used for day 6 cultures. In this manner, changes in fluorescence intensity could be detected. In the study of cytochalasin D effects, the film and print exposure times were the same as that noted for cultures prior to cytochalasin D treatment.

To illustrate concomitant observations of the G- and F-actin pools within the same endothelial cell, fluorescein DNase I ( $12\ \mu\text{M}$ ) and R-P ( $0.25\ \mu\text{M}$ ) were employed. The high concentration of DNase I was used to compensate for the rapid quenching of the fluorescein. Permeabilized BAEC were exposed to R-P for 10 min, washed with HBSS and then exposed to fluorescein DNase I for 10 min followed by another HBSS wash. The first image was acquired using a standard fluorescein filter set, while a second image of the same specimen was obtained using a standard rhodamine filter set. Collection and resolution of the fluorescent images were optimized using a Plan Neofluar  $100\times 1.3$  oil objective (Carl Zeiss, Oberkochen, Germany) and a cooled CCD Camera (model Star-1, Photometrics Ltd., Tucson, AZ).

The total exposure time for both images was approximately 4 sec, which minimized photobleaching and maximized contrast. The film was processed using an Image-1/AT System (Universal Imaging, West Chester, PA). With this system, the two digital images were re-registered to correct for slight optical filter misalignments and to adjust color perception so that the fluorescence of fluorescein and rhodamine appeared green and yellow-orange, respectively. The final photograph was produced on Ektachrome film (Kodak: 400 ASA).

## RESULTS

A comparison of the in situ G- and F-actin distributions in BAEC is illustrated in Fig. 1. G-actin appeared to be distributed diffusely, with enhanced fluorescence near the nucleus, such that this organelle was highlighted in the photographs (Fig. 1A). Fluorescence intensity associated with in vitro G-actin (Fig. 2, A-E) was greater than that observed under conditions in situ. However, distributions of G-actin were diffuse in both the BAEC in situ and in vitro. The fluorescence associated with in vitro G-actin was enhanced in the region of the cell nucleus and diminished in the cell periphery, which also demonstrated the similarities



**Fig. 1.** BAEC fixed in situ and exposed to R-DNase I (A) or R-P (B) to demonstrate the distribution of G- or F-actin, respectively. G-actin (A) was diffusely distributed and highlighted the area of the cell nucleus (n). F-actin was concentrated in the cell periphery and served to highlight the cell periphery. Some cells also showed thin F-actin stress fibers (arrows) that traversed the cell. Scale bar =  $10\ \mu\text{m}$ .

of the G-actin distributions in vitro and in situ. Other than the appearance of areas of bright punctate fluorescence, first noted on day 20 (Fig. 2D), and increased by day 32 (Fig. 2E), changes in the degree of BAEC confluency had little effect on the distribution of in vitro G-actin.

A low level of fluorescence was associated with in situ F-actin (Fig. 1B). F-actin was most concentrated in the cell periphery, casting an outline of the cell shape. Some cells also demonstrated the presence of fine stress fibers composed of F-actin that traversed the cell. In contrast, the F-actin fluorescence intensity was greatly increased for cultured cells (Fig. 2, F and G). Many F-actin stress fibers could be seen traversing the cells and in the cell periphery. Unlike in vitro G-actin, the in vitro F-actin distribution was dramatically altered by the degree of confluency. Subconfluent BAEC cultures (day 3; Fig. 2F) had cells with many stress fibers, while other cells had a more diffuse distribution with fewer stress fibers. Cultures on their first day of confluency (day 6; Fig. 2G) demonstrated intense fluorescence and numerous F-actin stress fibers traversed most cells. Four days postconfluency (day 10; Fig. 2H), there was an increase in the number of areas within the monolayer in which fluorescence intensity was diminished and the density of stress fibers was decreased. Fourteen days postconfluency (day 20; Fig. 2I), these areas were more prominent. Other than differences in their shape and size, BAEC now more closely resembled those seen in situ, since F-actin was generally noted in the cell periphery and few F-actin stress fibers could be demonstrated. At day 32 of culture (Fig. 2J), F-actin distribution was similar to that seen at day 10 or 20 of culture.

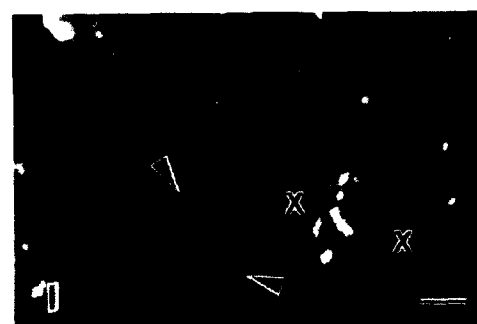
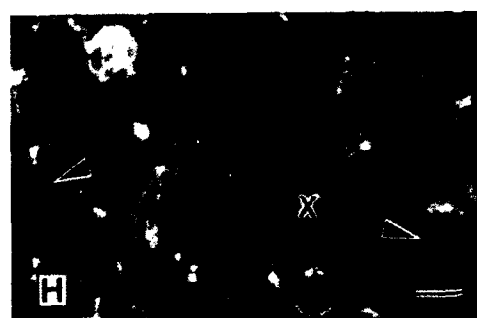
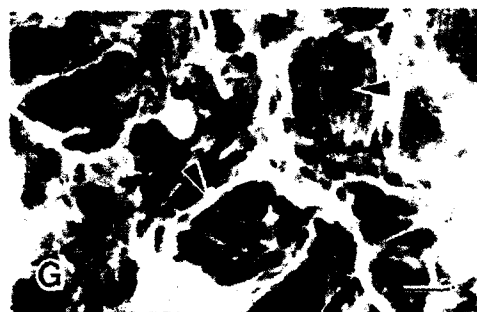
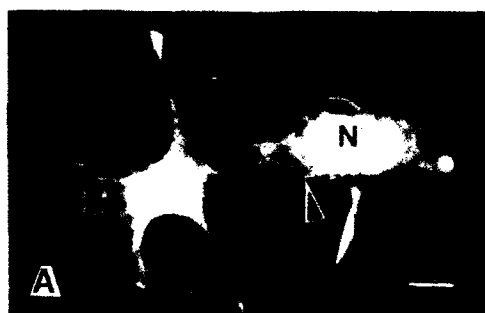
G-actin remained diffusely distributed and of similar fluorescence intensity after exposure to cytochalasin D (Fig. 3, A and B). This was in contrast to the effects of such exposure on the F-actin pool (Fig. 3, C and D). F-actin stress fiber networks were disrupted and there was an increase in diffuse and punctate fluorescence within the cells (Fig. 4D).

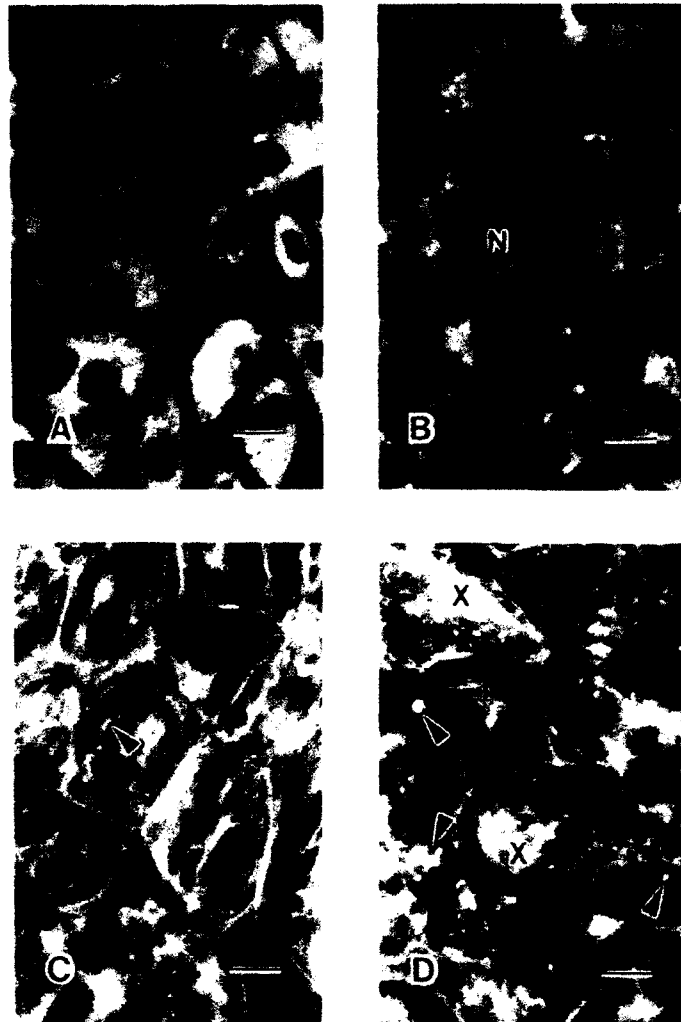
Simultaneous in vitro observation of the BAEC G- and F-actin pools is illustrated in Fig. 4. As noted when the actin pools were observed separately in different cells, the G-actin pool (green) appeared diffuse in nature and concentrated in the area of the nucleus, while F-actin (yellow-orange) was observed along the cell periphery and in stress fibers that traversed the cell.

## DISCUSSION

There is a paucity of qualitative information concerning F-actin distribution relative to that of G-actin. Although DNase I will interact with tropomyosin, this interaction is believed to be at a different site or to occur at a much reduced affinity compared to G-actin (Payne and Rudnick 1986). Moreover, tropomyosin does not prevent formation of the G-actin-DNase I complex (Hitchcock et al. 1976). DNase I has been used to quantitate actin (Blikstad and Carlsson 1982, Blikstad et al. 1978, Heacock et al. 1984, Heacock and Bamberg 1983a,b). This method relies, however, on the effectiveness of buffer systems to ensure that the actin polymerization state remains constant when the amount of actin comprising the F-actin pool is determined. The failure to maintain the actin polymerization state may be a source of error and account for some inconsistencies in quantitative findings (Heacock and Bamberg 1983b). Qualitative studies may aid not only our understanding of the interaction between the various pools of actin, but also may help to clarify quantitative data. The technique described here uses fluorescently labeled DNase I to visualize the G-actin pool relative to the F-actin pool.

As might be expected for an unstructured, monomeric form of a protein, G-actin distribution was diffuse both in situ and in vitro. As noted previously for in vitro F-actin (Rogers and Kalnins 1983b), the level of fluorescence associated with in vitro G-actin was greater than that observed for the in situ condition. This suggested that cultured cells have more G-actin. However, since these were qualita-





**Fig. 3.** Postconfluent BAEC (day 9) were treated (B and D) with cytochalasin D and then exposed to R-DNase I (G-actin probe; A and B) or R-P (F-actin probe; C and D). The G-actin pool was not affected by cytochalasin D, since both nontreated (A) and treated (B) cells demonstrated diffuse G-actin distribution and enhanced fluorescence surrounding the cell nucleus (N). Prior to cytochalasin D treatment (C), F-actin was distributed in numerous stress fibers that were located in the cell periphery or traversing the cell. In addition, some areas of punctate fluorescence (arrows) were noted. After treatment (D), stress fibers were disrupted and many areas of intense, diffuse (X) and punctate (arrows) fluorescence were seen. Scale bar = 10  $\mu$ m.

tive observations using different fixation procedures, G-actin quantitative methods on similarly treated samples must be stud-

ied to establish fully the differences in G-actin levels for the in situ and in vitro conditions. Although there was increased

**Fig. 2.** BAEC examined at various points of confluency under in vitro conditions were exposed to R-DNase I (G-actin probe; A–E) or R-P (F-actin probe; F–J) at 3 (preconfluent; A and F), 6 (confluent; B and G), 10 (4 days post confluent; C and H), 20 (14 days postconfluent; D and I) and 32 (26 days postconfluent; E and J) days of culture. G-actin distribution (A–E) was diffuse with the appearance of enhanced fluorescence near the nucleus (N). Areas of punctate fluorescence (arrows) were also noted, which increased in intensity by day 20 (D) and continued until by day 32 (E) most cells possessed a region of intense punctate fluorescence. The F-actin distribution (F) in some preconfluent cells (Y) was diffuse with few, if any, F-actin stress fibers, while in other cells (Z) such fibers (arrows) were more numerous. At confluency (G), cultures demonstrated intense fluorescence with numerous F-actin stress fibers (arrows) that were circumferential or traversed the cells. By day 10 (H), fluorescence intensity was diminished. F-actin stress fibers were fine, not as numerous and some cells (X) appeared to lack fibers that traversed the cell. At day 20 of culture (I) fluorescence intensity was also diminished. F-actin was mainly concentrated in the cell periphery (arrows) with many cells (X) showing low fluorescence in the cell interior. Fluorescence intensity and F-actin arrangement at day 32 of culture (J) were similar to that seen at day 10 or 20. F-actin was noted in the cell periphery (arrows) and most cells (X) lacked traversing F-actin stress fibers. Scale bar = 10  $\mu$ m.



**Fig. 4.** BAEC (day 3) were exposed to both R-P (F-actin probe) and fluorescein DNase I (G-actin probe). Cells were observed under fluorescence microscopy with image enhancement. The G-actin pool (green) was distributed diffusely around the cell nucleus. F-actin (yellow-orange) was located in the cell periphery and in stress fibers that traversed the cell. Thus, the G- and F-actin distributions under simultaneous observation within the same cell were similar to those noted when the actin pools were studied in separate cells.

fluorescence near the nucleus, this may not represent an area of increased G-actin concentration, since the region adjacent to the nucleus is of greatest cytoplasmic thickness and increased fluorescence would be expected when viewing such an area. Thus, although G-actin was visualized throughout the cell it was unclear if it actually existed in similar concentrations in the various cellular regions.

In contrast, the *in situ* arrangement of F-actin was concentrated in the region of the cell periphery and in stress fibers. This confirmed previous findings concerning the location of F-actin in endothelial cells studied directly from vessels (Gabbiani et al. 1983, Herman et al. 1982, Rogers and Kalnins 1983a,b, White and Fujiwara 1986, White et al. 1983, Wong et al. 1983).

When cultured under *in vitro* conditions, the F-actin arrangement is quite distinct from endothelial cells *in situ* (Rogers and Kalnins 1983b). As verified by the study reported here, there was an increase in F-actin stress fibers, which were located both circumferentially and traversing the cell (Fig. 2, F and G). However, with prolonged culture ( $\geq 10$  days; Fig. 2, H–J), the *in vitro* F-actin arrangement more closely approximated the *in situ* condition. This finding was relative to the shorter culture periods (day 6; Fig. 2G), since photographic exposure times for the long term cultures were the same as those used for the short term cultures. If automatic exposure times had been employed, the longer term cultures would have appeared to have had a greater fluorescence inten-

sity, which would not have accurately depicted the true F-actin distribution. Though this change (day 10; Fig. 2H) was not observed to be uniform, areas of reduced F-actin and stress fiber density did become more pronounced with time, such that by day 20 (Fig. 2I), this F-actin distribution was predominant and was still observed at day 32 (Fig. 2J). Quantitative studies indicated that F-actin levels can be reduced in some cell lines after obtaining a state of confluence (Heacock et al. 1984), which these qualitative findings support. Such data suggest that endothelial cells in culture might best be studied when maintained for a sufficient period to allow for an F-actin distribution more in keeping with that seen in the natural state.

In spite of notable changes in F-actin arrangement, the state of culture confluence had little observable impact on G-actin distribution. G-actin remained diffusely dispersed with only the appearance of enhanced punctate fluorescence noted after 20 days of culture (Fig. 2, D and E). Since its appearance (day 20) did not coincide with the first sign of a reduction in F-actin stress fiber density (day 10; Fig. 2H) and became more pronounced (day 32; Fig. 2E) in the absence of any further alterations in the F-actin pool (day 32; Fig. 2J), the enhancement of punctate fluorescence associated with G-actin appeared independent of the differences in F-actin distribution. Thus, while F-actin arrangement was dramatically altered by the degree of confluence, it did not appear to affect G-actin directly. Similarly, when cytochalasin D treatment was used to alter F-actin arrangement, no effect on the G-actin pool was observed (Fig. 3). This corroborated earlier quantitative findings that cytochalasin D treatment of human tumor cell lines does not result in net depolymerization of actin filaments (Morris and Tannebaum 1980). Thus, the appearance of increased diffuse and punctate F-actin fluorescence associated with cytochalasin D treatment (Fig. 3D) indicated that the F-actin of the disrupted stress fibers may have coalesced into these areas

and that they did not depolymerize to increase the G-actin pool.

In conclusion, alterations in the distribution of F-actin by the degree of confluence or cytochalasin D treatment did not appear to influence significantly qualitative differences in the G-actin pool. This suggests that whatever quantitative changes might have occurred in the G- and F-actin pools, alterations in the G-actin qualitative findings were unremarkable. Although changes in G-actin may be too subtle to observe due to the unstructured nature of this monomeric protein, these results suggested that F-actin alterations occurred independent of substantial effects on the G-actin pool. In addition, since modulations in F-actin arrangement and actin stress fiber density were more readily apparent than qualitative changes in G-actin, filamentous actin might be the principal factor in cellular functions influenced by actin cytoskeletal changes. As illustrated in Fig. 4, the simultaneous use of fluorescent probes for G- and F-actin in conjunction with image enhancement might serve to facilitate the investigation of the relative contributions made by both actin pools to cellular function.

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